Occurrence of Conjugated Polyenoic Fatty Acids in Seaweeds from the Indian Ocean

Narayan Bhaskar[a,b,*], Tomohisa Kinami[b], Kazuo Miyashita[b], Si-Bum Park[c], Yasushi Endo[c], and Kenshiro Fujimoto[c]

[a] Laboratory of Biofunctional Material Chemistry, Graduate School of Fisheries Science, Hokkaido University, Hakodate 041 8611, Japan. E-mail: bhasg3@yahoo.co.in
[b] Central Food Technological Research Institute (CFTRI), Mysore (India), presently a Monbusho Scholar at Hokkaido University, Hakodate 041 8611, Japan
[c] Graduate School of Agricultural Sciences, Tohoku University, Sendai 981 8555, Japan

* Author for correspondence and reprint requests

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Three species of red marine macro algae (Rhodophyta) from the Indian Ocean were analysed for the occurrence of conjugated polyenes. The composition of different lipid classes in these seaweeds along with their fatty acid composition has also been reported. Analysis of lipid classes of these seaweeds revealed that both *Acanthophora spicifera* (Ceramiales, Rhodophyta) and two species of *Gracilaria*, *viz. G. edulis* and *G. folifera* (Gracilariales, Rhodophyta) were rich in glycolipids followed by neutral- and phospholipids. The fatty acid composition of these seaweeds revealed C16:0 as the predominant fatty acid in all three species. However, *A. spicifera* had significantly higher amounts of eicosapentaenoic acid (EPA) and arachidonic acid (AA) as compared to negligible amount of these fatty acids in both species of *Gracilaria.* The red seaweed *Acanthophora spicifera* contained conjugated eicosapentaenoic acid (CEPA) and conjugated arachidonic acid (CAA) in all lipid classes except glycolipids.

Key words: Fatty Acid Composition, Conjugated Fatty Acids, Seaweeds

Introduction

The occurrence of polyunsaturated fatty acids (PUFAs) in general and η-3 fatty acids in particular is an unique feature of lipids of marine origin and the PUFAs have considerable health and economic significance (Lands, 1982; Lopez and Gerwick, 1987). The major components of plants in general such as proteins, carbohydrates, nucleic acids and lipids have been examined and explored for further processing as sources of human nutrition and/or pharmacological properties (Dembitsky *et al.*, 1990; Sardesai, 1992). Some studies have supported the role of conjugated fatty acids (CFAs) as bioactive molecules in the treatment of tumors and other cancer-related problems (Fritsche and Steinhart, 1998; Noguchi *et al.*, 2001; Kohno *et al.*, 2002) with varying degree of cytotoxic effects on the cancer cells (Suzuki *et al.*, 2001). There are several reports indicating the occurrence of conjugated dienoic, trienoic and tetraenoic fatty acids in plants (Takagi and Itabashi, 1981; Spitzer *et al.*, 1991) most of which are C_{18} compounds originating from oleic acid, linoleic acid, linolenic acid and stearidonic acid. Further, relatively simple biochemical modifications of arachidonic acid (AA) and eicosapentaenoic acid result in molelcules that possess important bioregulatory and hormonal functions (Gibson, 1982; Piper, 1986).

It has been reported recently that conjugated PUFAs such as conjugated eicosapentaenoic acid (CEPA), conjugated arachidonic acid (CAA) and conjugated docosahexaenoic acid (CDHA) prepared by alkali isomerisation had profound cytotoxic effects against human cancer cell lines (Matsumoto *et al.*, 2001). Though many workers have contributed to the information on the fatty acid composition of seaweeds from different regions of the world, not much information is available on the occurrence of the conjugated PUFAs in the seaweeds. In all, there are only four reports on the occurrence of these conjugated polyenes, *viz.* trienes in *Ptilota* (Lopez and Gerwick, 1987; Wise *et al.*, 1994; Park *et al.*, 2004a, b), tetraenes in *Bosiella orbingniana* (Burgess *et al.*, 1991), *Lithothamnion corallioides* (Hamberg, 1992) and *Anadyomene stellata* (Mikhailova *et al.*, 1995). The work on investigation of conjugated polyenes from

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the seaweed *Ptilota filicina* resulted in the definition of a polyenoic fatty acid isomerase (PFI) (Wise *et al.*, 1994) and the work on enzyme from *L. corallioides* explained the mechanism of formation of tetraene by that enzyme (Hamberg, 1992). PFI has recently been characterized and functionally expressed by DNA cloning (Zheng *et al.*, 2002). Further, not much work has been reported in the published literature regarding the fatty acid composition and/or occurrence of conjugated polyenes in seaweeds from the Indian Ocean areas. Hence, we were interested in examining the fatty acid composition of different species of seaweeds harvested from the waters of Indian Ocean with emphasis on the occurrence of polyenoic fatty acids. Our interest in this field is also a part of our evaluation of the biomedical potential of these bioactive molecules derived from marine lipids. In this communication we present the occurrence of some of the conjugated polyenes in seaweeds belonging to Rhodophyta, especially *Acanthophora spicifera*, from the Indian Ocean along with their fatty acid composition.

Materials and Methods

Plant materials

Three species, *viz. Acanthophora spicifera*, *Gracilaria folifera* and *G. edulis* (Rhodophyta, red seaweeds) were collected from the inter-tidal zones of Mandapam (Kerala), west coast of India. The seaweeds were washed in running water and shade dried at 35 ± 2 °C (to remove the surface moisture) before being frozen and transported to the laboratory. The samples were stored at – 30 °C until further use.

Extraction and isolation

The seaweeds (100 g) were first extracted in methanol (1:10 w/v) at room temperature by continuously stirring for 1 h using a magnetic stirrer. The extract was then filtered and the residue was successively re-extracted with methanol (1:10 w/v) chloroform/methanol (1:1 v/v) followed by chloroform (1:10 w/v). The total extracts were combined and solvents were evaporated under vacuum at 30 ± 1 °C. The dry green residue was re-dissolved in chloroform and phase separated with distilled water. The chloroform fraction was dried over anhydrous sodium sulphate to remove traces of moisture to get the lipid extract. This crude extract was first subjected to preparative TLC with hexane/diethyl ether/acetic acid (70:30:1 v/v/v) as the mobile phase.

Part of the lipid extract was used for analysis of the composition of different lipid classes. The lipid classes were separated by silica gel column chromatography (1:30 w/w of lipid) by successive elution with chloroform, acetone/methanol (9:1 v/v) and methanol to get neutral (NL), glyco- (GL) and phospholipids (PL), respectively.

All fractions along with the total lipids were transmethylated using sodium methoxide (0.5 M) to obtain the fatty acid methyl esters (FAMEs). FAMEs were also prepared by boron trifluoride (BF_3) method. These were further analysed by gas chromatography (GC) and reversed phase high performance liquid chromatography (RP-HPLC) using a photodiode array detector.

GC and HPLC analysis

GC: A Shimadzu GC-14B was used for fatty acid analysis. The GC was equipped with a FID and an Omega wax-320 fused silica capillary column (30 m × 0.32 mm i.d.). The detector, injector and column temperature were 260, 250 and 200 °C, respectively, with an analysis time of 100 min. The carrier gas was helium with a flow of 50 kPa.

HPLC: HPLC experiments were carried out with a Hitachi HPLC-D7000 equipped with a photodiode array detector. The columns used were LiChrocart RP18e (250 × 4.0 mm i.d.) for isolating the trienoic acid fractions and Devlosil C30 UG-5 (250 × 4.6 mm i.d.) at the time of identification using the standard CEPA and CAA. The mobile phase was methanol/water (85:15 v/v) in case of LiChrocart and acetonitrile/water (85:15 v/v) in case of Devlosil. The analysis temperature was 30 °C and 40 °C, respectively. The flow rate was 1.0 ml min^{-1} in both cases. The detector was set at 210, 233, 274 and 315 nm for detecting fatty acids, dienes, trienes and tetraenes.

Chemicals

All the chemicals and solvents used were of analytical grade. Solvents used for HPLC analysis were HPLC grade. The CEPA and CAA standards were prepared by enzymatic means using the crude polyenoic fatty acid isomerase (PFI) isolated from the seaweed *Ptilota pectinata* following the previously described methods (Lopez and Gerwick, 1987; Wise *et al.*, 1994). The enzymatically

Table I. Mean (± SD) lipid content (%, on dry wt. basis) and composition of lipid classes (% of total lipids) in different red algae (PL, phospholipids; GL, glycolipids; NL, neutral lipids; SD, standard deviation).

	*Acanthophora spicifera**	*Gracilaria folifera**	*G. edulis***
Lipid content			
	0.9 ± 0.15	0.8 ± 0.09	0.6 ± 0.11
Lipid class composition			
NL	26.3 ± 1.18	18.1 ± 0.56	20.6 ± 1.26
GL	63.0 ± 2.10	71.7 ± 2.98	71.7 ± 3.02
PL	10.9 ± 0.98	10.2 ± 1.05	7.7 ± 1.03

* n = 5; ** n = 4.

derived CEPA contained 69.6% 5c,7t,9t,14c,17c-EPA and 20.7% of 5t,7t,9t,14c,17c-EPA. Similarly, CAA contained 69.4% of 5c,7t,9t,14c-AA and 19.3% of 5t,7t,9t,14c-AA.

Results and Discussion

The amount of extracted lipids from each of the seaweed species is presented in Table I along with the composition of lipid classes. It was observed that the lipid content ranged from 0.6% (*G. edulis*) to almost 1% (*A. spicifera*) indicating that these seaweeds had a very low lipid content. This is in conformity with earlier reports (Heiba *et al.*, 1997; Kamenarska *et al.*, 2002) pertaining to other seaweeds. The fatty acid composition of total lipids (TL) and the lipid classes are presented in Table II. Arachidonic acid (AA, 20:4 n-6; 10.19% of total fatty acids) and eicosapentaenoic acid (EPA, 20:5 n-3; 6.18% of total fatty acids) formed the major fatty acids in *A. spicifera* apart from palmitic acid (16:0), which was the dominant fatty acid (40.27% in *A. spicifera* to more than 80% in *Gracilaria*) in all the three seaweeds. Such high contents of saturated fatty acids have earlier been reported in one of the warm water seaweeds (Hamdy and Dawes, 1998) and this can be attributed to the influence of environmental factors and/or characteristic feature of the individual genus (Khotimchenko, 1991). These results with regards to *A. spicifera* are in conformity with the earlier findings

Table II. Fatty acid composition (% of total fatty acids) of total lipids and different lipid classes of *Acanthophora spicifera* and *Gracilaria* sp. (TL, total lipids; NL, neutral lipids; GL, glycolipids; PL, phospholipids).

Fatty acid	*Acanthophora spicifera**				*Gracilaria edulis***				*Gracilaria folifera**			
	TL	NL	GL	PL	TL	NL	GL	PL	TL	NL	GL	PL
12:0	0.57	0.92	0.42	0.18	0.15	1.36	0.10	0.20	0.17	1.36	0.13	0.16
14:0	6.74	3.70	10.36	1.54	2.47	3.00	2.51	2.00	2.91	3.46	2.90	2.69
15:0	0.96	1.44	0.92	0.44	0.45	0.86	0.39	0.51	0.49	1.19	0.43	0.62
16:0	40.27	22.89	57.30	15.75	84.60	26.49	90.53	58.27	81.28	20.77	86.86	60.87
16:1 n-7	1.43	1.53	1.48	1.92	0.38	nd	0.23	3.17	0.47	2.53	0.18	3.62
16:2 n-4	0.23	0.40	0.16	0.16	nd	nd	nd	nd	nd	nd	nd	nd
16:3 n-4	0.38	0.70	0.26	0.24	nd	nd	nd	nd	nd	nd	nd	nd
18:0	1.43	2.00	1.21	1.30	1.24	1.57	1.05	2.30	1.51	1.83	1.36	2.36
18:1 n-9	10.21	11.46	11.22	4.40	0.71	3.35	0.35	1.90	1.00	3.56	0.61	1.93
18:1 n-7	2.18	1.65	1.91	3.43	0.69	0.68	0.43	4.48	0.81	0.85	0.60	5.52
18:1 n-5	0.20	0.27	0.18	0.12	nd	nd	nd	nd	nd	nd	nd	nd
18:2 n-6	1.37	1.93	1.06	0.92	0.16	0.49	nd	1.42	0.21	1.10	0.11	0.35
19:0	0.23	0.25	0.11	0.52	nd	nd	nd	nd	nd	nd	nd	nd
18:3 n-3	0.79	0.88	0.73	0.37	nd	nd	nd	nd	0.10	0.19	nd	0.63
20:1 n-11	0.16	0.29	bd	0.23	nd	nd	nd	nd	nd	nd	nd	nd
20:1 n-9	0.13	0.20	bd	0.12	nd	nd	nd	nd	nd	nd	nd	nd
20:2 n-6	0.20	0.29	0.12	0.18	0.12	0.15	0.11	0.40	0.27	0.26	0.30	0.45
20:3 n-6	0.48	0.59	0.31	0.66	0.23	nd	0.21	0.66	0.36	0.29	0.35	0.77
20:4 n-6	10.19	5.79	2.26	43.17	0.67	0.53	0.63	0.88	0.57	0.51	0.64	1.02
20:5 n-3	6.18	4.04	3.15	16.41	nd	nd	nd	nd	nd	nd	nd	nd
24:1 n-9	1.32	3.88	0.31	0.47	0.57	2.00	0.20	2.19	0.50	2.12	0.54	1.24
Others***	14.35	34.92	6.53	7.47	7.56	59.52	3.26	21.62	9.35	59.98	4.99	17.60
CFA	P	P	AB	P	AB	AB	AB	AB	AB	AB	AB	AB

CFA : Conjugated fatty acids including CEPA and CAA; P: present; AB: absent.
All values are a mean of four or more individual samples; * n = 5; ** n = 4; *** unidentified.
nd: Not detected.
bd: Low detection level (< 0.1%).

for the same species (Ramavat *et al.*, 1997). However, Ramavat *et al.* (1997) reported higher EPA (about 13%) and a very low AA content (about 1.7%) in the same species harvested from the Saurashtra coast, west coast of India. In the present investigation we found that both, AA and EPA, were present in relatively higher quantities (about 10.2 and 6.2% of the total fatty acids, respectively) with AA being higher than EPA. This can be attributed to the differences in climatic and geographical conditions from where the seaweeds were harvested. Apart from this, di-homo γ-linolenic acid (DGLA, 20:3 n-6) was found in reasonable quantities in all three species (from 0.2% in *Gracilaria* to about 0.5% in *Acanthophora*). This particular fatty acid has been reported to be the precursor for the synthesis of prostaglandin PGE1 (Groenewald and Van der Westhuizen, 1997) and a number of other related biologically active substances (Gerwick and Bernart, 1993).

Among the mono-unsaturated fatty acids (MUFAs), Δ9 isomers were predominant in both, *Acanthophora* and *Gracilaria*, with a particularly higher content in *A. spicifera* (>12% of the total fatty acids). In the decreasing order of abundance these were 18:1 n-9, 16:1 n-7, 24:1 n-9, 20:1 n-11 and 20:1 n-9 with the latter two present only in *A. spicifera.* The predominance of Δ9 isomers, especially in *Acanthophora*, suggests the presence of Δ9 desaturase which primarily acts on 18:0 and 16:0 acids (Khotimchenko, 1991). In our study we also found a MUFA, namely 24:1 n-9, with chain length more than C_{20}. In previous reports related to *Acanthophora* (Ramavat *et al.*, 1997), *Gracilaria* (Norziah and Ching, 2000) and other seaweeds (Wu *et al.*, 1995; Herbreteau *et al.*, 1997) fatty acids with a carbon chain length more than C_{20} has not been recorded except in brown seaweeds of *Sargassum* (Khotimchenko, 1991).

The HPLC analysis of the FAMEs of *A. spicifera* revealed the presence of conjugated trienes with distinct trident shaped spectra, with two shoulders, specific for trienes. However, both species of *Gracilaria* did not exhibit any absorbance at 274 nm indicating the absence of trienes. Further, the fractions containing the trident shaped spectra from *A. spicifera* were collected using HPLC. Two fractions containing the polyenes were collected and further purified by repeated HPLC. The FAMEs derived from total lipids and different lipid classes, were then compared with the CEPA and CAA obtained enzymatically using the enzyme from *Ptilota pectinata.* Fraction I contained two CEPA forms as revealed by comparison of spectroscopic data and retention times in HPLC with the standard CEPA and CAA. The CEPA forms that were present in the samples are 5c,7t,9t,14c,17c-EPA and 5t,7t,9t,14c,17c-EPA. Both forms had trident shaped peaks with absorption maxima at 263, 275 and 283 nm identically to that of the standard CEPA. Fraction II contained two distinct trident shaped peaks and was identified as conjugated arachidonic acid (CAA) on comparison with the standard. The CAA forms present in *A. spicifera* are 5c,7t,9t,14c-AA and 5t,7t,9t,14c-AA. It is very interesting to note that these conjugated polyenes were present in all lipid fractions except glycolipids. The presence of these in the neutral lipid fraction makes us to speculate that they could be present in free forms in this macro-alga. Further, the occurrence of these polyenes in only one of the three species of red seaweeds, although harvested from the same geographical area, let us to postulate that *A. spicifera* may contain some enzyme similar to that reported in *Ptilota* sp. (Wise *et al.*, 1994; Zheng *et al.*, 2002; Park *et al.*, 2004a, b) and/or *Bosiella orbigniana* (Burgess *et al.*, 1991).

In a recent study it was observed that CEPA and CAA obtained by alkali isomerisation showed a selectively higher cytotoxic effect on human cancer cell lines (Matsumoto *et al.*, 2001). We are further looking into the cytotoxic effects of these conjugated polyenes on different cancer cell lines, which will be reported in due course. Also, we wish to explore the fate of these conjugated polyenes in an *in vivo* model system through animal feeding experiments.

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